Rapid Detection and Identification of *N*-Acetyl-*L*-Cysteine Thioethers Using Constant Neutral Loss and Theoretical Multiple Reaction Monitoring Combined with Enhanced Product-Ion Scans on a Linear Ion Trap Mass Spectrometer

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A sensitive and specific liquid chromatography-mass spectrometry (LC-MS) method based on the combination of constant neutral loss scans (CNL) with product ion scans was developed on a linear ion trap. The method is applicable for the detection and identification of analytes with identical chemical substructures (such as conjugates of xenobiotics formed in biological systems) which give common CNLs. A specific CNL was observed for thioethers of *N*-acetyl-*L*-cysteine (mercapturic acids, MA) by LC-MS/MS. MS and HPLC parameters were optimized with 16 MAs available as reference compounds. All of these provided a CNL of 129 Da in the negative-ion mode. To assess sensitivity, a multiple reaction monitoring (MRM) mode with 251 theoretical transitions using the CNL of 129 Da combined with a product ion scan (IDA thMRM) was compared with CNL combined with a product ion scan (IDA CNL). An information-dependent acquisition (IDA) uses a survey scan such as MRM (multiple reaction monitoring) to generate "informations" and starting a second acquisition experiment such as a product ion scan using these "informations." Th-MRM means calculated transitions and not transitions generated from an available standard in the tuning mode. The product ion spectra provide additional information on the chemical structure of the unknown analytes. All MA standards were spiked in low concentrations to rat urines and were detected with both methods with LODs ranging from 60 pmol/mL to 1.63 nmol/mL with IDA thMRM. The expected product ion spectra were observed in urine. Application of this screening method to biological samples indicated the presence of a number of MAs in urine of unexposed rats, and resulted in the identification of 1,4-dihydroxynonene mercapturic acid as one of these MAs by negative and positive product ion spectra. These results show that the developed methods have a high potential to serve as both a prescreen to detect unknown MAs and to identify these analytes in complex matrix. (J Am Soc Mass Spectrom 2005, 16, 1976–1984) © 2005 American Society for Mass Spectrometry

The determination of metabolites of xenobiotics, which may serve as biomarkers of exposure, in blood and urine of humans is an analytical challenge since humans may be exposed to a variety of compounds at low concentrations [1]. Therefore, analytical methods have to be developed to screen blood or urine samples for unknown chemicals or known drugs and their metabolites for compound identification to calculate ultimate exposure levels [2]. Over recent years, tandem LC-MS/MS has been extensively applied to quantify compounds with known structures using transitions for the sensitive multiple reaction monitoring (MRM) methods [3].

Many LC-MS/MS methods to determine specific metabolites of xenobiotics have been described [4]. These hydrophilic analytes are well ionized by electrospray. However, the compound-specific methods do not permit screening for compounds with unknown structures such as metabolites, and synthetic reference materials are necessary to develop a sensitive MRM method in complex matrices [5–8]. Metabolite stan-

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dards are rarely available and screening for unknowns in the full scan mode is difficult owing to the limited sensitivity and specificity in complex matrices. Therefore, the aim was to develop a screening method to detect unknown compounds that carry identical substructures. This approach permits the preselection of an analyte group [9]. The preselection criterion may be a specific product ion such as *m/z* 113 in glucuronides, [10] a specific neutral loss such as 162 Da involving a glucose substructure as present in glycosides [9], or the neutral loss of 129 Da of glutathione metabolites [11]. The choice of transition determines the choice of the survey scan mode. With a conventional tandem MS, a product ion scan can only be performed in a second chromatographic separation and, in addition, the instrument is less sensitive than ion trap instruments [12, 13]. Therefore, the combination of a tandem mass spectrometer and a sensitive ion trap in a single instrument is the instrument of choice for precursor ion scan (PreIS) or CNL combined with the acquisition of product ion mass spectra [14, 15].

The new generation of linear ion traps offers the possibility to combine CNL, PreIS, or MRM with a product ion scan on a linear ion trap to acquire a mass spectrum of every signal detected within one single separation, since the third quadrupole is constructed as an ion trap [16]. In addition to the specificity of the survey scans, the product ion spectrum provides additional data to elucidate the structure of unknowns or to confirm known analytes [17]. Glucuronic acid or sulfuric acid conjugates and mercapturic acids (MAs) are prominent metabolites of xenobiotics formed by biotransformation. MAs are formed by reaction of electrophiles with glutathione and are eliminated in urine after transpeptidase cleavage [18]. Due to the possible presence of many glucuronides, sulfates, and mercapturic acids in the complex matrix, the availability of additional spectral information is important. Moreover, isobaric compounds may give false-positive results. In this study, standards of mercapturic acids were analyzed for characteristic product ions to generate specific CNL or PreIS scans. In addition, theoretical MRM transitions (thMRM) were generated using the CNL observed to compare sensitivity to the IDA CNL method. For MRM, CNL, thMRM, and the corresponding IDA methods, sensitivity and specificity were compared and applied to biological samples from experiments to generate MAs under different conditions.

Materials and Methods

Chemicals

Unless otherwise noted, all chemicals were purchased from Sigma/Aldrich (Taufkirchen, Germany) and were of the highest purity available. *N*-acetyl-(2,2-dichlorovinyl)-*L*-cysteine, *N*-acetyl-(1,2-dichlorovinyl)-*L*-cysteine, *N*-acetyl-(4-chlorobenzyl)-*L*-cysteine, *N*-acetyl-(2-fluorobenzyl)-*L*-cysteine, *N*-acetyl-(4-bromobenzyl)-*L*-cysteine, *N*-acetyl-(4-methoxybenzyl)-*L*-cysteine, *N*-acetyl-(4-tert-butylbenzyl)-*L*-cysteine, *N*-acetyl-(1,1-dichloro-2,2-difluoroethyl)-*L*-cysteine [19], *N*-acetyl-(1,2,2-trichlorovinyl)-*L*-cysteine, *N*-acetyl-(3-chloropropenyl)-*L*-cysteine, and *N*-acetyl-S-purinyl-*L*-cysteine were synthesized and characterized as described previously [20, 21]. *N*-acetyl-3-OH-propyl-*L*-cysteine, *N*-acetyl-phenyl-*L*-cysteine, and *N*-acetyl-(2,4-dinitrophenyl)-*L*-cysteine were obtained from Toronto Research Chemicals (North York, Canada). 3-(*N*-acetylcystein-S-yl)-acetaminophen, 3-*N*-acetyl-(1,4-dihydroxynonenyl)-*L*-cysteine [22], and rat liver microsomes were kindly supplied by F. Hoffmann-La Roche (Basel, Switzerland).

Synthesis of N-*Acetyl-S-(2-Carbamoylethyl)-L-Cysteine*

This adduct was synthesized according to the *N*-acetyl-3-oxopropyl-*L*-cysteine synthesis of Ramu et al. [23] using acrylamide in place of acrolein and characterized by comparison of recently published mass spectral data [24].

Instrumentation

The HPLC system consisted of an Agilent quaternary solvent pump and an Agilent Autosampler Series 1100 (Waldbronn, Germany). The MS/MS system consisted of a TURBO-Ionspray source and a QTRAP mass spectrometer API 2000 (Applied Biosystems, Darmstadt, Germany). The centrifuge used was an Eppendorf Centrifuge 5415C (Hamburg, Germany). A Synergi polar RP18 4 μ (150 $\times$ 2 mm i.d.), a Synergi hydro RP18 4 μm (150 $\times$ 2 mm i.d.) from Phenomenex (Aschaffenburg, Germany), or a Reprosil Pur C18 Aq 5 μm (150 $\times$ 2 mm i.d.) from Dr. Maisch GmbH (Ammerbruch, Germany) were used. The precolumn (C18 material, >3 mm i.d.) was also purchased from Phenomenex.

Analytical Methods

Stock solutions of the MA standards (1 mg/mL in MeOH) were diluted with water. MS parameters such as declustering potential (DP) or collision energy (CE) were optimized with the "quantitative optimization" function of analyst 1.3.2 or 1.4 to generate a MRM method optimized for the individual MA standard method. This MRM method was used for the optimization of the HPLC parameters like flow rate, solvents, and gradient. All standards were directly introduced into the TURBO-IonSpray source using 5 mM ammonium acetate, pH 6.8, 5 mM ammonium formiate, pH 3, or 0.1% formic acid as mobile phases. A syringe pump provided a constant analyte flow into the LC eluent (0.25 mL/min) by a T-connection. Optimal sensitivity was obtained with ammonium acetate (5 mM, pH 6.8) in the negative-ion mode. HPLC-separations were performed with 5 mM ammonium acetate, pH 6.8 (Solvent A) and acetonitrile (AcCN) (Solvent B) with a flow rate

Table 1. Important MS parameters used for the detection of the standards in the negative-ion mode

Method	DP [V] used	CE [V] used	Dwelltime [ms]
Compound optimized MRM	DP	CE	
N-acetyl-(1,2-dichlorovinyl)-*L*-cysteine	−31	−12	1000
N-acetyl-(2,2-dichlorovinyl)-*L*-cysteine	−51	−20	1000
N-acetyl-(4-chlorobenzyl)-*L*-cysteine	−56	−16	1000
N-acetyl-(2-fluorobenzyl)-*L*-cysteine	−41	−20	1000
N-acetyl-(3-hydroxypropyl)-*L*-cysteine	−71	−20	1000
N-acetyl-(4-brombenzyl)-*L*-cysteine	−51	−24	1000
N-acetyl-(4-methoxybenzyl)-*L*-cysteine	−76	−22	1000
N-acetyl-(4-tert-butylbenzyl)-*L*-cysteine	−76	−26	1000
3-(*N*-acetylcystein-S-yl)-acetaminophen	−56	−20	1000
N-acetyl-(1,1-Dichloro-2,2-difluoroethyl)-*L*-cysteine	−61	−16	1000
N-acetyl-phenyl-*L*-cysteine	−71	−22	1000
N-acetyl-(2,4-dinitrophenyl)-*L*-cysteine	−26	−16	1000
N-acetyl-(1,2,2-trichlorovinyl)-*L*-cysteine	−51	−8	1000
N-acetyl-(3-chloropropenyl)-*L*-cysteine	−66	−20	1000
N-acetyl-S-(2-carbamoylethyl)-*L*-cysteine	−41	−28	1000
N-acetyl-(S-purinyl)-*L*-cysteine	−21	−24	1000
IDA MRM (251 masses)	−50	−20	5
IDA CNL (251 masses)	−50	−20	3000
CNL with four experiments	−50	−20	each exp.: 1000
CNL with one experiment	−50	−20	3000

of 0.25 mL/min using a mixture of MA standards in a concentration of 8.3 μg/mL each. A gradient with the following conditions was used: initial conditions were 5% Solvent B followed by a linear gradient from 5 to 50% B in 16 min, then 50% B for 4 min, 50 to 100% B in 1 min, 100% B for 2 min, 100 to 5% B in 1 min, and re-equilibration by 5% B for 6 min. All HPLC columns tested showed good peak shapes and a sufficient separation of the standards but different retention times.

CNL and Optimized MRM

Standards were scanned for the CNL of 129 Da in the negative-ion mode with the following parameters: source voltage, −4.2 kV; vaporizer temperature, 400 °C; curtain gas, 30 psi; nebulizer gas, 45 psi; turbogas, 50 psi; CAD gas, medium. When using the positive-ion mode, only the source voltage was changed to 5.5 kV. The CNL method development consisted of four experiments with various DPs and CEs: experiment 1, DP −50 V, CE −20 V; experiment 2, DP −30 V, CE −30 V; experiment 3, DP −40 V, CE −40 V; experiment 4, −50 V, CE −40 V. Collision Entrance Potential (CEP) and Collision Cell Exit Potential (CXP) were set to −10 V and −2 V, respectively. Q1 resolution was set at "unit", Q3 resolution was set at "low". Differences in MS parameter settings are described in Table 1.

IDA CNL and IDA thMRM

Survey scans were performed with either a CNL of 129 Da (mass range of 251 Da) or thMRM with 251 theoretical mass transitions, adapted to the expected mass ranges. Pause between the mass ranges was set to 5 msec in the CNL mode and 2 msec in the thMRM mode. The information-dependent acquisition experiments were performed with the following settings: if signals in the survey scan exceeded 500 cps, a product ion scan was performed; if more than one signal was recorded at the same time, the highest signal was selected. Former target ions were then excluded for at least 40 s. The mass tolerance was set to 250 mmu. Fragments formed in the product ion scan were detected in the range between *m/z* 50 and 500 with dynamic fill time, a scan rate of 4000 u/s, and resolution of Q1 was set to unit.

Determination of LOD in Solvent and Biological Matrix

For the determination of the LOD of the MAs with the IDA methods, a mixture of 12 MA standards with concentrations of 0.04, 0.08, 0.16, 0.2, 0.4, 0.8, 1.6, and 4.2 μg/mL of the individual analytes was diluted 1:2 with either urine, incubation medium, or MeOH/H_2O (50/50 vol/vol). *N*-acetyl-(4-methoxybenzyl)-*L*-cysteine (10 μg/mL) was used as the internal standard. Urine was collected from Wistar rats housed in Macrolon cages [6]. Urinary proteins were precipitated by adding AcCN (0 °C) to the urine sample (50/50 vol/vol) followed by centrifugation at 15,000 × *g* for 20 min. The supernatant was removed and the procedure was repeated. The organic phase was removed under a stream of nitrogen. Incubation solutions were prepared as described below for controls. After 30 min of incubation at 37 °C, the standard mixture was added. The sample preparation used for the incubation media was identical to that described for urine samples. Aliquots of each sample (5 μL) were analyzed with the IDA CNL and the IDA thMRM method (n = 5). The LOD was defined as

the concentration of analyte yielding the recording of at least three out of five product ion scans and showing a signal with a CNL of 129 Da and S/N (signal to noise) ratio of the corresponding peak heights of three.

Incubations with Diclofenac

Diclofenac was incubated at 37 °C for 60 min in a shaking water bath with liver microsomal proteins from rats. Incubation volume was 500 μL and consisted of 0.1 M potassium phosphate buffer (pH 7.4), 1 mM magnesium chloride, 10 mM glucose-6-phosphate, 1 mM NADP, 0.5 U/mL glucose-6-phosphate-dehydrogenase, protein (1 mg/mL), and 10 mM *N*-acetyl-*L*-cysteine (*N*-acys). Diclofenac (final concentration of 50 μM or 100 μM) was added after 5 min of preincubation. Incubations without substrate served as controls. Reactions were terminated by the addition of AcCN (500 μL). After centrifugation, the supernatant was removed, and again 500 μL of cold AcCN was added. The mixture was centrifuged and the organic phase was removed by a stream of nitrogen. Aliquots (50 μL) were injected into the LC-MS/MS system. For the metabolism assay, the mass range used was adapted to the expected molecular masses of *m/z* 472 with a scan from *m/z* 250 to 500. Samples were injected three times with each method and mean values of the resulting peak areas and S/N ratios were used to compare the sensitivity of IDA CNL and IDA thMRM.

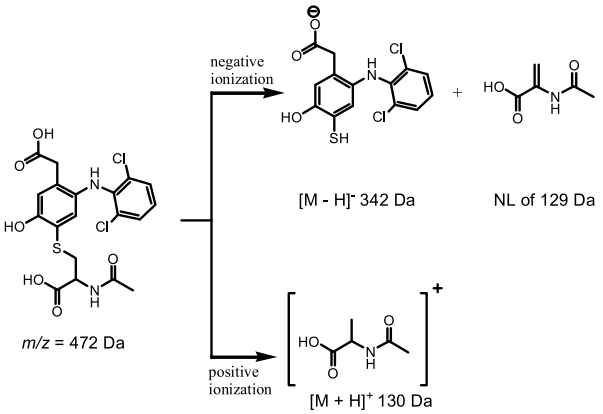
Results and Discussion

Development of IDA Methods

A linear ion trap offers different scan modes to record a product ion scan of each signal detected by the survey scan. The intensities of both PreIS and CNL may be similar when identical scan times are used. However, precursor ion scan may be more sensitive compared to a CNL if a dominant product ion is formed for the same analyte and vice versa (i.e., if a dominant neutral loss occurs such as loss of 162 Da for glycosides [25]). For matrix samples (plasma or urine), a theoretically generated multiple reaction monitoring scan (thMRM) may be even more sensitive compared to CNL and PreIS as survey scans, because the noise level is significantly reduced. This needs to be considered for the selection of the type of survey scan applied to obtain highest sensitivity and selectivity.

Based on these considerations, mass spectra of different thioethers of *N*-acetyl-*L*-cysteine were recorded to obtain characteristic substructure fragmentation patterns. In the negative-ion mode, all available standards showed a CNL of 129 Da, resulting from the cleavage of the thioether (Scheme **1**).

In the positive-ion mode, a product ion of *m/z* 130 (the corresponding fragment that resulted from the CNL in the negative-ion mode) and/or a CNL of 163 Da



Scheme 1. Fragmentation of the mercapturic acid of diclofenac by collision induced dissociation (CID) in the negative and positive-ion mode. The neutral loss (NL) of 129 Da in the negative-ion mode was observed in all mercapturic acids tested so far. With positive CID, diclofenac showed a fragment ion of 130 like other standards, but did not show a CNL of 163 Da.

(the *N*-acetyl-*L*-cysteine moiety) was observed in the product ion spectra of many of the standards (Scheme **1**). However, a common fragment or a CNL did not occur in the positive-ion mode. These observations are consistent with the reported spectra of mercapturic acids derived from styrene, 1,3-butadiene, acrolein, diclofenac, valproic acid, and dopamine, where CNLs of 129 Da occur in the negative-ion mode and a fragment ion of *m/z* 130 is formed in the positive-ion mode [8, 26–30].

Several compound-specific parameters relevant to the generation of mass spectra were optimized with "quantitative optimization". Declustering potential and collision energy were varied from −21 to −76 V and from −8 to −28 V, respectively, for the available standards (Table 1). To determine a common DP and CE for highest average sensitivities for all standards, DP and CE were set from −10 to −30 V for CE and −30, −50, and −70 V for DP, respectively. With a DP of −50 V and CE of −20 V, all standards were detected with satisfying sensitivity. For thMRM, the CNL of 129 Da was used to calculate theoretical transitions (i.e., *m/z* 200 → 71).

Optimization of the HPLC Parameters for the MA-Screening Method

The chromatographic separation should provide optimized S/N ratios combined with high ionization efficiency to achieve optimized sensitivity with LC-MS. Ammonium acetate (5 mM, pH 6.8) was found to be the optimal buffer to detect the standards with peak shapes and separation efficiency with a full width at half maximum ($b_{1/2}$) ≤ 0.14 min on the RP-18-HPLC columns used (Figure 1).

Comparison of Sensitivity

The LOD of each MA was different depending on the chemical structure and ranged from 0.58 to 140 pmol on

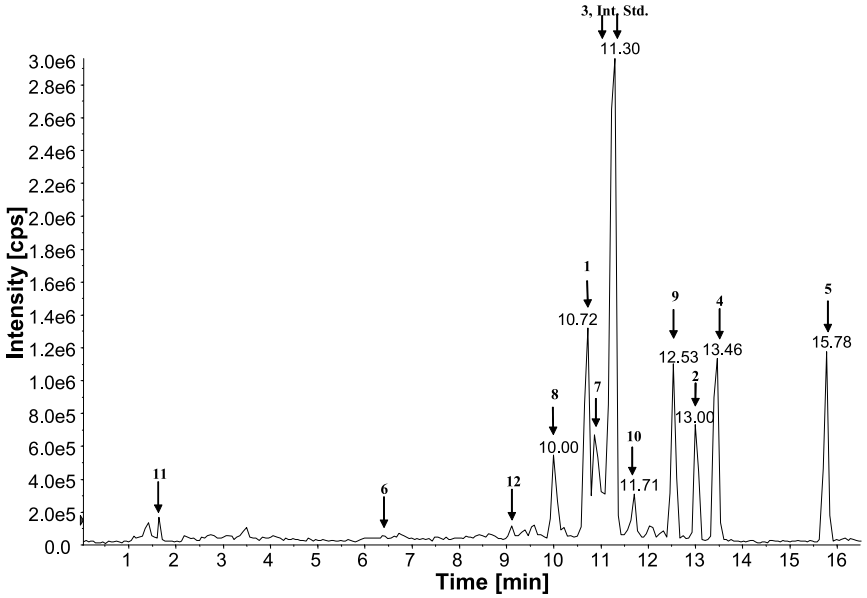


Figure 1. LC-ESI-MS/MS chromatogram of a separation of twelve mercapturic acid standards (1–12, see Table 2), each with a concentration of 2.5 μg/mL and an internal standard of *N*-acetyl-(4-methoxybenzyl)-*L*-cysteine, spiked to rat urine (injection volume of 5 μL). The chromatogram was obtained with IDA CNL. Two of the mercapturic acids elute at t_R = 11.73 min.

column in the CNL with a mass range from *m/z* 200 to 450. The LOD in the MRM mode with one transition optimized for each MA was 100- (*N*-acetyl-(4-bromobenzyl)-*L*-cysteine) to 4400-fold (*N*-acetyl-(1,1-dichloro-2,2-difluoroethyl)-*L*-cysteine) lower than in the CNL mode due to optimized parameters, noise reduction, and an increase in scan time in the MRM mode.

However, for the detection of unknown MA, an optimization is not possible. Therefore, methods were developed with mass spectrometric and HPLC parameters applicable to analyze unknown MA with DP of −50 V and CE of −20 V. A mass range from *m/z* 200 to 450 was set for CNL and thMRM. CNL was compared with IDA CNL, thMRM for 251 transitions (thMRM-251) with IDA thMRM-251, respectively, with thMRM-25. The CNL methods were compared with the corresponding thMRM method to evaluate each method for specificity and sensitivity. The S/N ratios were slightly higher (1.2-fold) in CNL compared to IDA CNL and 1.3-fold higher in thMRM compared to IDA thMRM. S/N ratios of thMRM-25 compared to S/N ratios of thMRM-251 were about 5-fold higher. S/N ratios of thMRM-251 compared to CNL were about 2-fold higher. A comparison of CNL with a mass range of only 25 Da i.e., from *m/z* 200 to 225 with thMRM-25, was not performed since the molecular masses of the standards ranged from *m/z* 221 to 332 and showed a disadvantage of the CNL scan (data not shown).

Compared to the data in MeOH/H_2O, the LOD in urine samples were comparable for both methods for 10 of the 12 standards. For *N*-acetyl-(1,2-dichlorovinyl)-*L*-cysteine and *N*-acetyl-3-OH-propyl-*L*-cysteine, the LOD was 5-fold lower in the IDA CNL and 2-fold lower in the IDA thMRM-251 compared to MeOH/H_2O matrix (Table 2). For all standards, the IDA thMRM-251 was equal to or up to 2-fold more sensitive as compared to IDA CNL. In spiked samples from microsomal incubations, the LODs of the MA standards were in the same range (from 2-fold lower to 5-fold higher) as described for urine and MeOH/H_2O samples (data not shown). The mercapturic acid of acetaminophen was used to determine the linearity with r^2 = 0.997 (IDA CNL) and r^2 = 0.998 (IDA thMRM-251) in a dynamic range of 0.3 μg/mL to 100 μg/mL in rat urine samples.

In summary, both methods applied were equally sensitive in all matrices tested. The different LOD for the standards in different matrices may be explained by the influence of the sample solvents containing buffers and salts on the electrospray ionization of standards eluting close to the solvent front (e.g., *N*-acetyl-(3-hydroxypropyl)-*L*-cysteine with a retention time of 1.7 min; Figure 1).

Application of the Method to Detect Derivatives of N-*Acetyl*-L-*Cysteine in Biological Samples*

Urine samples of rats not intentionally exposed to xenobiotica were screened for the presence of mercapturic acids with both IDA methods. Over 50 signals in the CNL 129 Da survey scan and the thMRM survey scan, respectively, were observed (Figure 2). Intensity varied form sample to sample. Product ion spectra of the corresponding precursor ions were recorded. However, in all samples analyzed, a compound with the $[M-H]^-$ mass of 320 Da was present (Figure 3a). The product ion spectra (positive- and negative-ion mode, Figure 3b and c) of this compound were identical to

Table 2. LOD [μg per mL] of standard mercapturic acids spiked to rat urine and to MeOH/H_2O (50:50 vol./vol.), respectively. Detection of the signals by IDA CNL and IDA thMRM. The LOD was defined as the concentration of analyte yielding the recording of at least three out of five product ion scans and giving a signal (S/N ratio > 3) with a CNL of 129 Da

	LOD [μg/mL] determined with IDA CNL		LOD [μg/mL] determined with IDA MRM	
Compounds	Rat urine	MeOH/H_2O	Rat urine	MeOH/H_2O
N-acetyl-(2,2-dichlorovinyl)-*L*-cysteine (1)	0.04	0.04	0.02	0.02
N-acetyl-(4-chlorobenzyl)-*L*-cysteine (2)	0.02	0.04	0.02	0.02
N-acetyl-(2-fluorobenzyl)-*L*-cysteine (3)	0.04	0.04	0.04	0.04
N-acetyl-(4-bromobenzyl)-*L*-cysteine (4)	0.02	0.02	0.02	0.02
N-acetyl-(4-tert-butylbenzyl)-*L*-cysteine (5)	0.02	0.02	0.02	0.02
3-(*N*-acetylcystein-S-yl)-acetaminophen (6)	0.42	0.42	0.21	0.42
N-acetyl-(1,1-Dichloro-2,2-difluoroethyl)-*L*-cysteine (7)	0.10	0.21	0.10	0.21
N-acetyl-phenyl-*L*-cysteine (8)	0.04	0.02	0.04	0.02
N-acetyl-(2,4-dinitrophenyl)-*L*-cysteine (9)	0.02	0.02	0.02	0.02
N-acetyl-(1,2,2-trichlorovinyl)-*L*-cysteine (10)	0.10	0.21	0.10	0.21
N-acetyl-(3-hydroxypropyl)-*L*-cysteine (11)	0.21	0.83	0.10	0.21
N-acetyl-(1,2-dichlorovinyl)-*L*-cysteine (12)	0.42	2.08	0.42	0.83

spectra of the synthesized standard of 1,4-dihydroxynonene mercapturic acid. Hence, this compound was identified as the mercapturic acid of 1,4-dihydroxynonene with $[M - H]^-$ at 320 Da, an endogenous metabolite of 4-hydroxynonenal formed during lipid peroxidation [22, 31].

Diclofenac was chosen as a model substrate to test the suitability of the analytical approach to detect mercapturic acid formation from metabolites of xenobiotics formed by microsomal oxidations. The structures of these derivatives formed from diclofenac are known [30, 32]. In incubations of diclofenac with microsomes from humans and rats in the presence of *N*-acetyl-*L*-cysteine, two metabolites with $[M - H]^-$ ions at *m/z* 471 were detected by both IDA CNL and IDA thMRM-251 analysis (Figure 4). By product ion analysis, the signals with *m/z* 471 were identified as two mercapturic acids of hydroxydiclofenac (data not shown). The fragmentation of these ions resulted in the formation of product ions at *m/z* 162 and 342. The fragment ion at *m/z* 162 was assigned to the *N*-acetyl-*L*-cysteine moiety, whereas the fragment ion at *m/z* 342 resulted from the neutral loss of 129 Da. The ion of *m/z* 298 was formed by the combined loss of 129 and of 44 Da (CO_2). The fragment with *m/z* 262 is probably afforded by an additional loss of HCl. In addition, the chlorine pattern could be used for confirmation of the presence of a diclofenac metabolite.

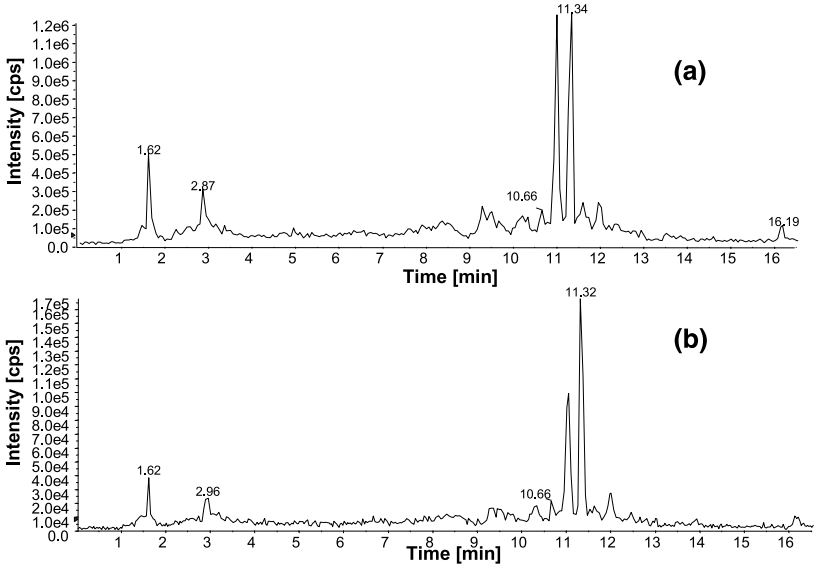


Figure 2. Comparison of total ion current (TIC) chromatograms of the methods IDA CNL (**a**) and IDA thMRM (**b**) in control rat urine for the detection of endogenous and unknown mercapturic acids. Chromatogram of a selected mass is achievable by extracting this mass from the TIC.

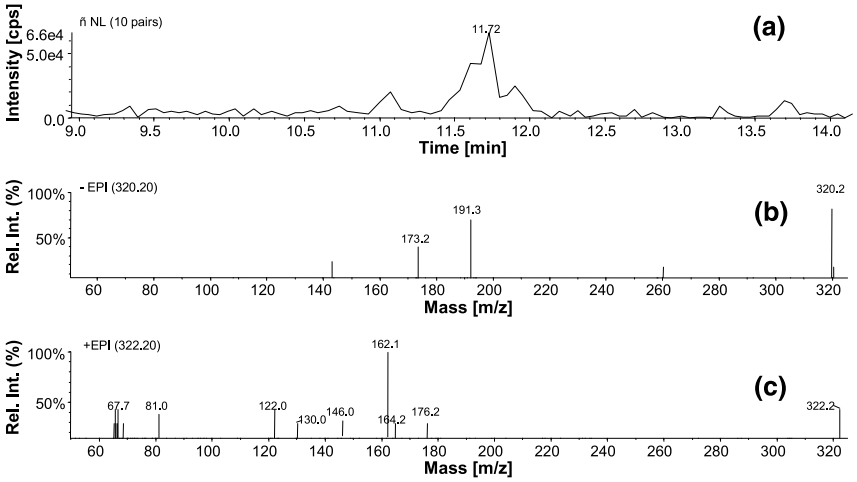


Figure 3. Extracted ion current (XIC) of ten masses (*m/z* 315 to 324) in IDA CNL (**a**) in a separation of a rat urine sample. The product ion spectra of the metabolite with *m/z* 320 in the negative-ion mode (**b**) and the corresponding mass of *m/z* 322 in the positive-ion mode (**c**).

Evaluation of Both IDA Methods

Both IDA methods were applicable to all matrices tested. One advantage of the IDA thMRM may be the applicability of two different transitions typical for one single analyte to trigger the recording of product ion spectra (i.e., two different CNL, two different product ions, or more probably the combination of a CNL and a specific product ion). The possible disadvantage of using non-optimized collision energies would be overcome by the use of two different collision energies (e.g., by the addition of another experiment with a higher CE to the method) to generate different mass spectra with different fragment intensities (data not shown).

Moreover, dwell times have an influence on the sensitivity of the thMRM method. Therefore, scanning the masses with a dwell time higher than 5 ms will result in a further increase in sensitivity. In tests, an increase of the dwell time to 15 ms already gave a 2-fold higher S/N ratio. With an approximate knowledge-based prediction of the metabolite of interest, the mass window may be restricted, the dwell time may be extended, and sensitivity may be increased as shown for the comparison of IDA thMRM-251 and IDA thMRM-25.

Conclusions

The presented data show the capabilities of modern mass spectrometry for detection and identification of analytes like mercapturic acids in complex matrices. Both IDA methods, utilizing CNL or thMRM, show similar sensitivity to screen for thioethers of *N*-acetyl-*L*-cysteine in microsomal incubations or urine samples. The slightly lower sensitivity of IDA methods com-

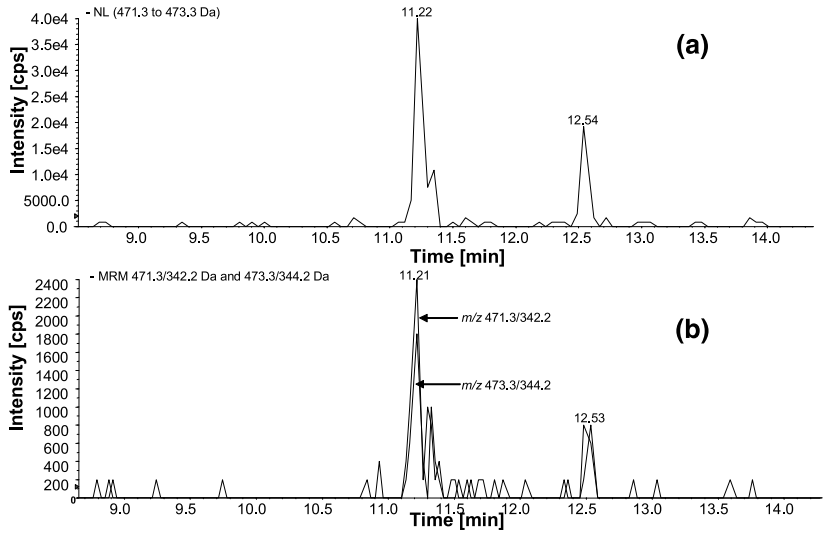


Figure 4. Extracted ion current chromatogram of two mercapturic acids of hydroxydiclofenac with a mass of *m/z* 471 (and the isotope with *m/z* +2) obtained with IDA CNL (**a**) and IDA thMRM (**b**).

pared to methods without IDA may be improved by a more efficient sample preparation (i.e., sample enrichment). However, the advantage of additional information on chemical structure of the detected compounds by IDA could be considered as more important compared to the slightly higher sensitivity without IDA.

The presented methods may be applied to other groups of analytes providing a CNL or a special product ion that could be used for a precursor ion scan to present a survey scan in a corresponding IDA method. For example, DNA-modifications may be analyzed by this MS-MS technique using the loss of 116 Da (i.e., for etheno-dA or OTA-dG [33, 34]. The loss of 116 Da corresponds to the desoxyribose moiety comparable to the fragmentation described for glycosides by Qu et al. [25]. Another fragment of sugar compounds is formed by glucuronic acids (neutral loss of 176 Da) [6, 10].

IDA thMRM for known analytes may provide high sensitivity combined with high specificity in analytical methods. In one separation, an analyte can be quantitated and identified by retention time, MRM, and stable isotope labeled internal standard. Additional specificity is gained by recording the corresponding characteristic product ion mass spectrum of each signal detected by the MRM scan. This has recently been shown for 301 drugs [14].

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